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Regulation of Gut Gene Expression by Thyroid Hormone Receptor Variants

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Abstract

Thyroid hormone (T_3) plays a critical role in gut development and homeostasis. T_3 action is mostly mediated through its nuclear receptors, TRα1, TRβ1, and TRβ2, the major *bona-fide* TR isoforms that carry both the DNA and hormone binding domains. However, it has become clear that several TR variants also exist, notably TR$\Delta\alpha$1 and TRα2, each lacking the DNA and/or hormone binding domains. The present studies were undertaken to define the role of these TR variants in the context of a T_3-regulated gut-specific gene, intestinal alkaline phosphatase (IAP), an enterocyte differentiation marker that limits fat absorption. Transient transfections of Caco-2 cells with an IAP-luciferase reporter plasmid carrying the 2.5 kb IAP proximal promoter showed that the TR variants inhibited TRα1-mediated activation of the IAP gene. Quantitative real-time RT-PCR showed that these non-*bona-fide* TRs were also able to inhibit endogenous IAP gene activation by the TR. As expected, EMSA confirmed that TRα2, but not TR$\Delta\alpha$1, binds to the IAP-TRE. We conclude that the two naturally occurring TR variants (TR$\Delta\alpha$1 and TRα2) repress TRα1-mediated activation of the IAP target gene through distinct mechanisms. These results indicate that the physiological effects of T_3 in the gut occur through a complex interplay among both activating and inhibitory TR isoforms.

Introduction

Thyroid hormone (T_3) plays a crucial role in intestinal epithelial development and homeostasis (20, 56). Prior to weaning there is a dramatic increase in T_3 levels associated with a growth spurt within the gut, and a shift in enterocyte gene expression from an immature to a mature phenotype (13). In addition to the effects T_3 has during weaning, adult hypothyroid animals display marked crypt villus hypoplasia, and revert to the suckling pattern of brush border enzyme expression, i.e. high lactase and low IAP levels (4, 16, 33, 39, 50, 55). Studies in knockout mice have confirmed the critical role that T_3 plays in gut development and homeostasis. Most notably, T3 receptor (TR)α knockout mice display significant intestinal abnormalities with marked epithelial hypoplasia, decreased levels of various digestive enzymes, and impaired B- and T-lymphocyte proliferation (2, 7).

Gene regulation by T_3 is mediated through its nuclear receptors (TR) that interact with thyroid hormone response elements (TRE) in target genes. Binding of T_3 to the TR activates or inhibits the T_3-responsive genes, depending upon interaction with a variety of co-activator and co-repressor proteins (57). The TREs usually consist of a loosely conserved hexamer (AGGTCA) repeat intervened by four nonspecific nucleotides, also known as direct repeat 4 (DR4) (6, 49). TRs can also bind to other forms of these two hexameric repeats, such as palindrome and everted repeats (40). TRs bind to TREs with highest affinity as a TR-RXR heterodimer complex, which serves as the primary biological mediator of T_3 action (28, 57). TRs, like other nuclear receptors, contain five structurally similar, independent, but functionally-related domains (45). Starting from the N-terminus, these domains are organized as, (i) modulator or A/B domain, (ii) DNA-binding domain (DBD) or C-domain, (iii) hinge region or D-domain, (iv) ligand-binding domain (LBD) or E-domain, and (v) F-domain. The TRs containing both DBD and LBD are known as *bona-fide* TR isoforms, and include TRα1, TRβ1 and TRβ2.

On the other hand, when a TR lacks the DBD and/or LBD, it is recognized as a non-*bona fide* TR, or TR variant, e.g., TRα2 (lacking LBD) and TRΔα1 (lacking both DBD and LBD).

The human TRs are encoded by two different genes located on chromosomes 17 and 3, encoding TRα and TRβ, respectively. The TRα locus gives rise to the *bona-fide* TRα1 isoform and at least two additional TR variants, TRΔα1 and TRα2 (10, 25). TRΔα1 is a truncated 155 amino acid TRα isoform that contains neither DNA binding nor ligand binding sites (5, 10). TRΔα1 is produced from a small transcript that is generated from a promoter located within intron 7 of the TRα1 gene (5). Studies in TRΔα1 knockout mice have revealed the delicate balance that exists between TRα1 and TRΔα1 in regard to normal gut development (38). TR-/- mice, in which the TRα1 and TRα2 genes are deleted but TRΔα1 transcripts are expressed, display a significant impairment in gastrointestinal development (38). These mice also suffer from severe hypothyroidism and succumb to death shortly after weaning (7). Mice which lack both TRα1 and TRΔα1 (TRα1-/-) were reported to exhibit lower body temperature, bradycardia, but had a normal life span (51) indicating the potential importance of either TRΔα1 or TRα2. In the TR 0/0 knockouts, where the entire TRα locus is deleted, there was only moderate impairment in gut development, mild hypothermia, and normal life span suggesting that the excessive TRΔα1 activity is responsible for the severity of the phenotype in TR-/- mice (38). TRΔα1 knockouts have mild alterations in enterocyte phenotype, but the mice were more responsive to T_3 rescue compared to wild type mice (38).

The precise mechanism of TRΔα1 action is not entirely clear. In transfections using an artificial TRE-containing reporter gene, it has been shown that TRΔα1 interferes with the transcriptional activating functions of TRα1 and RXRα. In addition, TRα1 appears to trigger the proteasome-mediated degradation of TRΔα1, presumably counteracting the inhibitory activity of

over-expressed TRΔα (5). TRΔα1 may also inhibit other transcription factors involved in the process of enterocyte differentiation (38).

TRα2 is another non-*bona fide* splice variant of the TRα gene, a 490 amino acid protein that contains an N-terminal DNA binding domain (23, 25) and a unique C-terminus that does not bind to T_3. TRα2 is expressed in a wide variety of tissues, including the gut (27, 41, 46). *In vitro* studies have shown TRα2 to be a dominant negative inhibitor of T_3 action by competing for the DNA binding site (TRE) (3, 22, 26). We have previously shown that small intestinal TRα2 levels decline by approximately 90% at the time of weaning in post-natal rats, coinciding with the tissues becoming responsive to exogenous T_3 (17). These results suggest that the inhibitory effects of TRα2 may explain the fact that the gut is unresponsive to T3 during the suckling phase. TRα2 knockouts show no gastrointestinal abnormalities, but display a mixed hypothyroid and hyperthyroid phenotype (42).

Previous studies on the TR variants (TRΔα1 and TRα2) have utilized artificial TRE-containing reporter constructs rather than an actual T_3 target gene. As such, we sought to determine the relative roles of the TR variants in regulating the expression of intestinal alkaline phosphatase (IAP), a previously identified gut T_3 target gene (31). IAP is a brush border enzyme that is exclusively expressed in differentiated villus enterocytes and serves as a marker for enterocyte differentiation (15). Functionally, the IAP protein is a component of the surfactant-like -particles that are seen in enterocytes after fat ingestion (12, 21, 29). IAP knockout mice have been shown to gain more weight than wild type mice when fed on a high fat diet (35) and also display higher serum triglycerides after fat gavage. Given this role of IAP, we have speculated that the decreased dietary fat absorption seen with hyperthyroidism could be due to the loss of IAP expression that occurs in this setting (11, 48).

Our previous studies have shown that T_3 induces endogenous IAP expression through a TR-RXR complex that binds to a unique TRE located between -632 and -612 in the IAP

promoter sequence (31). This unique TRE consists of an everted repeat of two nonamers spaced by three nonspecific nucleotides. In this study, we show that T_3-induced TRα1-mediated IAP activation in the human colorectal adenocarcinoma Caco-2 cell line is inhibited both by TR$\Delta\alpha$1 and TRα2 in a dose dependent manner. TRα2, but not TR$\Delta\alpha$1, binds to the unique IAP-TRE. These results provide a description of how TR variants affect the expression of a specific target gene.

MATERIALS AND METHODS

Materials

T_3 and the SYBER Green JumpStart Taq ReadyMix kit for quantitative real-time PCR were obtained from Sigma (St. Louis, MO). DNA restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). Taq DNA polymerase and TNT^R T7 Quick Coupled Transcription/Translation System were bought from Promega (Madison, WI). Trizol reagent for RNA preparation and SuperScript III Platinum Two-Step qRT-PCR kit were obtained from Invitrogen (Carlsbad, CA). SuperFect transfection reagent, the kit for DNA extraction from agarose gel, and the kit for large scale DNA preparation were obtained from Qiagen (Valencia, CA). Radionucleotides were purchased from PerkinElmer Life Sciences (Boston, MA), and oligonucleotides were synthesized by Sigma Genosys (The Woodlands, TX). Poly(dI-dC).Poly(dI-dC) was obtained from Pharmacia (Piscataway, NJ).

Plasmids

We constructed the mammalian promoter-detection vector pFRL7, a derivative of the mammalian promoter-detection vector pFRL2 (30) that lacks the CMV promoter. We cloned the 2.6 kb *Kpn* I-*Nar* I fragment from the plasmid pIAP-2574/-49 (31), carrying the human IAP promoter region (-2574 to -49, relative to translation initiation codon AUG) into pFRL7 thus generating the full-length IAP-luciferase reporter plasmid pFRL7-IAP-2574. The human TRα1 (pSG5-TRα1) and RXRα (pSG5-RXRα) expression plasmids were the derivatives of the pSG5 vector (Stratagene, Cedar Creek, TX), and were the kind gifts of Anthony Hollenberg (Beth Israel Deaconess Medical Center, Boston, MA). TR$\Delta\alpha$1 was constructed by deleting 255 amino acids

from the N-terminus of the TRα1 protein. Briefly, the reverse primer (TRα1.57R: 5'–gaa gcg gcc gcc cag gat gcc ctc cag cac gcc aag aga c–3') was synthesized that was complementary to the sequence exactly upstream of the extreme 5' ATG in the cDNA sequence for TRα1 (underlined). The forward primer (TRα1.838F: 5'-cat gcg gcc gcc acc atg gag atc atg tcc ctg cgg gcg gct gtc-3') was synthesized containing the DNA sequence (underlined) that was equivalent to the amino acids 256 to 265, while the 256th amino acid being a methionine, and preceded by a Kozak sequence (CCACC). Both the primers carry the Not I restriction site at the 5' end. The primers were then used on pSG5-TRα1 to PCR-amplify the target DNA fragment containing the TRΔα1 nucleotide sequence as well as the vector (pSG-5) DNA sequence. The PCR fragment was then extracted with phenol-chloroform, digested with Not I restriction enzyme and recircularized by T4 DNA ligase. Competent DH5α E. coli cells were transformed and plasmid DNA from transformants was isolated. A plasmid containing the expected restriction sites was designated as pTRΔα1 (pSG5-TRΔα1). Partial DNA sequencing of pTRΔα1 showed correct sequence equivalent for the complete TRΔα1 protein.

Cell culture

The human colorectal adenocarcinoma-derived Caco-2 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) and was maintained in Dulbecco's modified Eagle's media (DMEM, Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cells were grown at 37^{o}C in the presence of 5% CO_2 and were split by trypsinization when they reached about 80-90% confluence. Ion-exchange resin and charcoal treatment was used to 'strip' off thyroid

hormone (T_3) from FBS. DMEM containing 10% 'stripped' FBS was used for studying effects of T_3 on the target cells (43)

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from untransfected Caco-2 cells as well as cells tranfected with plasmids expressing TRα1, TRα2 and/or TR$\Delta\alpha$1. Trizol reagent from Invitrogen Life Technologies (Carlsbad, CA) was used to prepare RNA following the manufacturer's instructions. For first strand cDNA synthesis SuperScript III Platinum Two-Step qRT-PCR kit from Invitrogen (Carlsbad, CA) was used. First strand cDNA was synthesized from individual samples of RNA isolated from untransfected and transfected cells. Briefly, the RNA sample (1 μg) was incubated with oligo$(dT)_{20}$ (2.5 μM) and SuperScript III reverse transcriptase in presence of dNTPs (400 μM) at room temperature for 15 min, followed by incubation at 42^{o}C for 90 min. The reverse transcriptase activity was terminated by treating the samples at 85^{o}C for 5 min. PCR was performed on the synthesized cDNA (20ng) with gene specific primers (0.2 μM) using Taq DNA polymerase (2.5 U) from Promega (Madison, WI). The PCR conditions were: first denaturation step at 94^{o}C for 2 min, then 32 cycles of 94^{o}C for 1 min (denaturing), 55^{o}C for 1 min (annealing), and 72^{o}C for 1 min (extension), followed by a 5 min final extension step at 72^{o}C. PCR products were electrophoresed in 2% agarose gel containing 0.025 μg/ml ethidium bromide. Gels were photographed under UV light using Gel Doc 2000 Gel Documentation System from Bio-Rad (Hercules, CA). The list of primers used in RT-PCR is given below:

1. hIAPcDNA2146F: 5'-gca acc ctg caa ccc acc caa gga g-3'
2. hIAPcDNA2423R: 5'-cca gca tcc aga tgt ccc ggg ag-3'
3. hTRα1TR$\Delta\alpha$1F: 5'-ggg caa cgt gct ggt tat tgt gct g-3'
4. hTRα1.1530R: 5'-cac act gtc ctt tcc ata gca agt tc-3'

5. hTRα2.1153F: 5'-ccg cac ttc tgg ccc aag ctg ctg atg-3'

6. hTRα2.1620R: 5'-cca gct ttc agg cac ctc ctg ctc ttg-3'

7. hβ-Actin601F: 5'-ggg tct gga cct ggc tgg ccg gga cct g-3'

8. hβ-Actin1100R: 5'-ggg ccg ccg atc cac acg gag tac ttg c-3'

PCR- amplification with the primers hIAPcDNA2146F and hIAPcDNA2423R generates a 278 bp IAP cDNA fragment. Primers hTRα1TRΔα1F and hTRα1.1530R amplify a 1530 bp TRα1 and a 735 bp TRΔα1 fragments. The TRα2 fragment produced by hTRα2.1153F and hTRα2.1620R is a 468 bp fragment. The control β-actin primers hβ-Actin601F and hβ-Actin1100R produces a 500 bp fragment.

Quantitative real-time PCR

Real-time PCR was performed on cDNAs to quantitate the amount of IAP expression in cells transfected with plasmids expressing TRα1, TRΔα1, and/or TRα2. The SYBER Green JumpStart Taq ReadyMix kit for quantitative real-time PCR was obtained from Sigma (St Louis, MO) and used following the manufacturer's protocol. The primers and conditions for real-time PCR were the same as described above for RT-PCR. Real-time PCR was performed in the DNA Engine Opticon 2 System (MJ Research, Waltham, MA).

Transient transfection and luciferase reporter assays

Transient transfection and luciferase reporter assays were performed following the protocols as previously described (31). Caco-2 cells were plated at a density of 300,000 cells per well of a 6-well plate in DMEM containing 10% 'stripped' FBS. Cells were grown overnight,

and transient transfections were performed using SuperFect reagent from Qiagen. Cells were treated +/- 100 nm T_3. Approximately 1.5 μg of a test plasmid DNA per well was used in each transfection. The total amount of DNA was kept the same for each transfection by adding nonspecific plasmid TF12 DNA. Firefly and *Renilla* luciferase assays were then performed on cell lysates using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 24 h later as per the manufacturer's instructions. The control *Renilla* luciferase activity was used to determine transfection efficiency as well as to normalize the firefly luciferase activity as a percentage of the *Renilla* luciferase activity. The activation ratio (fold-activation) was determined by dividing the normalized firefly luciferase counts in the presence and absence of T_3 (T_3^+/T_3^-).

In vitro protein synthesis

TNT^R T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) was used for *in vitro* synthesis of human TRα1, TRΔα1, TRα2 and RXRα proteins from the derivatives of pSG-5 (Stratagene) carrying the relevant coding sequences under the control of the T7 promoter.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were performed following the previously described protocol (31). Complementary oligonucleotides carrying the IAP-TRE (Top strand: 5'-ACTTTT GAACTCAGCCTGAGGTTACCAAACT-3', nonameric everted repeat underlined) were annealed in the presence of 100 mM NaCl. The double-stranded oligonucleotides were then radio-labeled by the kinasing reaction with T4 polynucleotide kinase in the presence of [γ-^{33}P]ATP. The radiolabeled probe was purified twice by passing it through Micro Bio-Spin 6 chromatography

columns (Bio-Rad, Hercules, CA), followed by determination of the specific activity, which usually measured about 10^8 cpm/μg DNA (31). Radiolabeled probe (10 ng) was incubated at room temperature for 20 min with 1 μl (~1 ng) of synthesized TRα1, TRΔα1, TRα2, and/or RXRα in 10 μl of binding buffer containing 20 mM HEPES (pH 7.7), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 5 μM nonspecific oligo, 10% glycerol, and 2 μg of Poly(dI-dC).Poly(dI-dC). The samples were electrophoresed in a 5% polyacrylamide gel in a cold room (4°C), followed by drying of the gel and autoradiography.

Results

TRΔα1 and TRα2 inhibit TRα-mediated transactivation of the IAP-luciferase reporter gene

Expression of both TRα1 and TR variants (TRΔα1, TRα2) in intestinal epithelia prompted us to investigate their relative roles in the regulation of the IAP gene. To determine the effects of TR variants on T_3-induced IAP activation, we transfected Caco-2 cells with the IAP-luciferase reporter plasmid pFRL7-IAP-2574 as well as with plasmids expressing the various TR proteins. The results are shown in Figure 1A. Consistent with our previously published data, T_3 treatment of Caco-2 cells caused an approximate 3-fold activation of the IAP gene by endogenous TRs (31). Both TR variants inhibited IAP gene activation by approximately 50%. Transfection of cells with the TRα1 expression plasmid caused an approximate 8-fold increase ($p<0.05$) in IAP-luc expression by T3, also consistent with our previously published data (31). As expected, co-transfection with the TRΔα1 or TRα2 expression plasmid also resulted in a dramatic inhibition of the exogenous TRα1-mediated IAP activation.

TRΔα1 and TRα2 do not inhibit Cdx1-mediated IAP activation

To determine the specificity of the effects of the inhibitory TR variants, we examined their effects on another known IAP transcriptional activator, Cdx1. We performed co-transfections with the Cdx1 expression plasmid in conjunction with the TRα2 or TRΔα1 expression plasmid. The results (Fig. 1B) showed an approximate 4-fold increase in Cdx1-mediated IAP activation, consistent with our previous data (1). This increase in Cdx1-mediated IAP activation was not inhibited by either TRΔα1 or TRα2 (Fig. 1B) indicating that the inhibitory effects of the TR variants are specific for the TRα1 pathway.

Inhibition of TRα1-mediated IAP transactivation by TR variants is dose-dependent

We next determined the dose-response effects of TR variants on TRα1-mediated transactivation of the IAP gene. We used varying amounts of TR$\Delta\alpha$1 and TRα-2, maintaining a constant amount (1.5 μg) of TRα1 in the transfection assays. The results show that both TRα2 and TR$\Delta\alpha$1 suppressed TRα1-mediated activation of the IAP gene in a dose-dependent manner (Fig. 2A & B, respectively). The relative activities of the TR variants appear to be similar and at a high concentration (4.5 μg) both TR variants completely abolish the transactivation effects of TRα1.

TR$\Delta\alpha$1 does not exert ligand-independent repression

Transfection of cells with TRα1 expression plasmid shows ligand-independent repression of the IAP gene due to overexpressed exogenous TRα1 (Fig. 3), consistent with the known effects of unliganded TR (18,19). In contrast, there was minimal repression of basal IAP gene expression with TRα2, and TR$\Delta\alpha$1 exerted no ligand-independent repression.

TR$\Delta\alpha$1 and TRα2 inhibit TRα1-mediated endogenous IAP gene activation

Preconfluent Caco-2 cells were transiently transfected with TRα1, TR$\Delta\alpha$1, and/or TRα2 expression plasmids, the cells were treated with or without 100 nm T_3, and total RNA was isolated 48 h later. Semiquantitative RT-PCR showed low or nonexistent levels of the TR variants in the untransfected cells, but as expected with co-transfection, high levels of the TR variants was confirmed (Fig 4A, middle panels). Consistent with our previous data (31), basal IAP levels were low, but increased significantly with T_3 treatment and were even higher with

TRα1 co-transfection (lane 2 and 4, respectively, upper panel). Co-transfection with the TR variants revealed that TRΔα1 and TRα2 both significantly inhibited TRα1-mediated activation of the endogenous IAP gene (lane 10 and 12, respectively, upper panel). The control β-actin bands indicate equal cDNA concentration in all samples (lower panel).

We then performed quantitative real-time PCR on the above-mentioned cDNA samples. The real-time PCR assays (Fig. 4B) confirmed the semiquantitative RT-PCR results, with low basal IAP levels and significant increases with TRα1 (14-fold, $p < 0.05$). TRα1-mediated activation of IAP expression was dramatically inhibited by TRΔα1 or TRα2 co-transfection (approximately 80% and 60%, respectively).

The IAP-TRE binds to the in vitro-synthesized human TRα1 and TRα2 proteins, but not to TRΔα1 protein

We next employed EMSA to determine whether the identified IAP-TRE is capable of binding TR isoform proteins *in vitro*. We synthesized a double-stranded oligonucleotide corresponding to the IAP-TRE sequence (31). EMSAs were performed using the radiolabled TRE probe and *in vitro*-synthesized TRα1, TRα2, or TRΔα1 proteins. Figure 5 demonstrates that none of the proteins alone bind to the IAP-TRE. However, as expected, both TRα1 and TRα2 efficiently bind to the TRE in the presence of RXR, thus generating shifted bands (lanes 6 and 7, respectively). These data are consistent with our previous work that characterized this TRE within the IAP gene (31). Importantly, TRΔα1 does not bind to the IAP-TRE (lane 8). These results show that TRα1 and TRα2, but not TRΔα1, bind to the novel IAP-TRE, suggesting distinct mechanisms for inhibition of TRα1-mediated activation of the IAP gene by these two TR variants.

Discussion

Thyroid hormone (T_3) is recognized as an important regulator of intestinal growth, development, and differentiation. Hypothyroid animals show marked hypoplasia of crypts and villi, and abnormally retain the suckling pattern of gene expression (13, 16). In the gut, T_3 action is primarily mediated by the TRα locus as demonstrated by knockout mice that showed marked abnormalities in function and structure of the of the intestine (7, 9, 37). The TRα locus encodes the TRα1 receptor and a few variants, including TRα2 and TR$\Delta\alpha$1. As a *bona-fide* receptor, TRα1 has both the DNA and hormone (T_3) binding domains. The TRα2 receptor has a DNA binding domain but lacks a hormone binding domain, whereas the TR$\Delta\alpha$1 variant lacks both domains, and as such these variants are known as non-*bona fide* TR isoforms (10, 23).

There has been a growing recent interest in the roles of the TR isoforms in regulating T_3 action in the small intestine. In regard to TRα2, its decreased expression with weaning could explain why the gut becomes responsive to exogenous T3 administration at that developmental time. TR$\Delta\alpha$1 is expressed in the mouse small intestine (38), and probably plays a role in modulating T_3 action in the gut. In knockout mice where the entire TRα locus is deleted, a less severe intestinal phenotype is observed compared to mice where TRα1 and TRα2 are abolished. This observation suggests that the unbalanced action of TR$\Delta\alpha$1 is responsible for the severe phenotype observed in these latter animals (38). In TR$\Delta\alpha$1 knockouts with normal expression levels of TRα1 and TRα2 there is enhancement of T_3-mediated proliferation suggesting that TR$\Delta\alpha$1 physiologically down-regulates the growth promoting effects of T_3. TR$\Delta\alpha$1 has also been shown to repress Cdx1 transcription, another gene involved in gut development (38). *In vitro* studies using an idealized, unnatural TRE have shown that TR$\Delta\alpha$1 inhibits T_3 action (5), but no previous studies have been reported regarding the effects of TR$\Delta\alpha$1 on an actual T3 target gene.

The human IAP gene maps to chromosome 2q34-37 and produces a membrane bound 528 amino acid polypeptide that hydrolyzes a wide variety of monophophate esters at high ph and is expressed most abundantly in the proximal small intestine (32). IAP is exclusively expressed in the villus enterocytes making it a well-established marker for crypt villus differentiation. IAP levels have been shown to increase at weaning in rats when other functional and structural changes are taking place in the gut that coincide with the thyroid hormone surge (15). Functionally, IAP appears to play an inhibitory role in the process of dietary fat absorption (35). In addition to the TR, IAP is transcriptionally regulated by a variety of other transcription factors associated with gut development, including KLF4, Cdx1, and HNF-4 (1, 14, 36, 44). Given the physiological significance of the IAP protein in modulating fat absorption and as a marker for differentiation, we chose IAP as the target gene for these studies to decipher the effects of the TR variants.

In the present study, we used transient transfections to show that IAP gene activation by TRα1 was inhibited by both TR$\Delta\alpha$1 and TRα2 in a dose-responsive manner (Figs. 1 & 2). Importantly, these effects of the two TR variant isoforms were specific for the TR pathway, since there were no inhibitory effects in regard to Cdx1 (Fig. 1B), a homeobox protein that plays a key role in intestinal homeostasis and is a known activator of IAP transcription (1, 8). Using semiquantitative and quantitative real-time RT-PCR (Fig. 4), we confirmed that expression of the endogenous IAP gene is increased 14-fold ($p<0.05$) after TRα1 transfection and that this induction is inhibited by TR$\Delta\alpha$1 and TRα2, approximately 80% and 60%, respectively ($p<0.05$) (Fig. 4B).

The TR$\Delta\alpha$1 variant does not appear to function in the absence of T3, as opposed to the TRα1 which blocks IAP expression via the process of ligand-independent repression (Fig. 3) (19, 57). Interestingly, TRα2 has been shown to be defective in corepressor recruitment (47) and it is unclear what role, if any, it plays in ligand independent repression (18). Minimal ligand-

independent repression has been observed in the case of TRα2-mediated regulation of artificial TREs (47). RXR heterodimerization with the C-terminus of TRs has been shown to recruit corepressors (58), and the absence or mutation of part of the C-terminus (compared to TRα1) has been thought to be responsible for inefficient recruitment of corepressors resulting in minimal ligand-independent repression observed in the case of TRα2. Consistent with this model, we observed minimal ligand-independent repression of the IAP gene with overexpressed TRα2. The TRΔα1 variant appears to exert its inhibitory effects only in the context of the T_3-bound TR, since we saw no ligand-independent repression with TRΔα1.

The distinct mechanisms of action of the TR variants was confirmed by the EMSA studies, showing that TRα1 and TRα2 both bind to the IAP-TRE in the presence of RXRα, whereas TRΔα1 does not bind to this TRE (Fig. 5). TRα2 inhibits TRα1 activity by direct competition for TRE binding (22). Interestingly, TRα2 heterodimerizes with RXR, and binding to DNA varies depending on the TRE in question. For example, the TRα2-RXR heterodimer can bind to a DR4 TRE but not to a palindromic or inverted repeat TRE (3, 22, 34, 52, 53). This binding pattern may represent a gene-specific mechanism for modulating T_3 action. We have previously defined the IAP-TRE as an everted repeat of two nonamers separated by three nonspecific nucleotides (31). We now show that TRα2-RXRα can bind to this novel DNA cis-element (Fig. 5). As such, the IAP-TRE is the first example of an everted repeat TRE that binds to the TRα2-RXRα heterodimer. The EMSA results are consistent with previous data suggesting that the mechanism of inhibition by TRα2 is via competitive binding to the TRE. The inability of TRΔα1 to bind to the IAP-TRE concurs with the previous findings that showed TRΔα1 does not bind to a TRE (5). Accordingly, we believe inhibition of the TRα1 effects on IAP by TRΔα1 is likely mediated via interference of the activating functions of TRα1 and RXRα, as suggested by Chassande et al. (5).

Because of the involvement of IAP in fat absorption, it will be of interest in the future to determine whether the levels of the TR and the two variant forms are changed by dietary factors or in models of obesity. Another potential functional role for the IAP protein may be as a host defense factor, since it has been shown to detoxify bacterial lipopolysaccharides (LPS) (24). Given the fact that T3 appears to ameliorate the systemic effects of sepsis in animal model systems (54), it will be interesting to examine a possible role for the various TR isoforms and IAP in the context of sepsis.

In conclusion, we have shown inhibition of TRα1-mediated activation of the IAP gene by non-*bona fide* TR isoforms *in vitro*. This is the first example of action of these inhibitory TR isoforms on a naturally occurring, endogenous T_3 target gene. These results could have important implications for the physiological modulation of T_3 action in the contexts of gut development and homeostasis.

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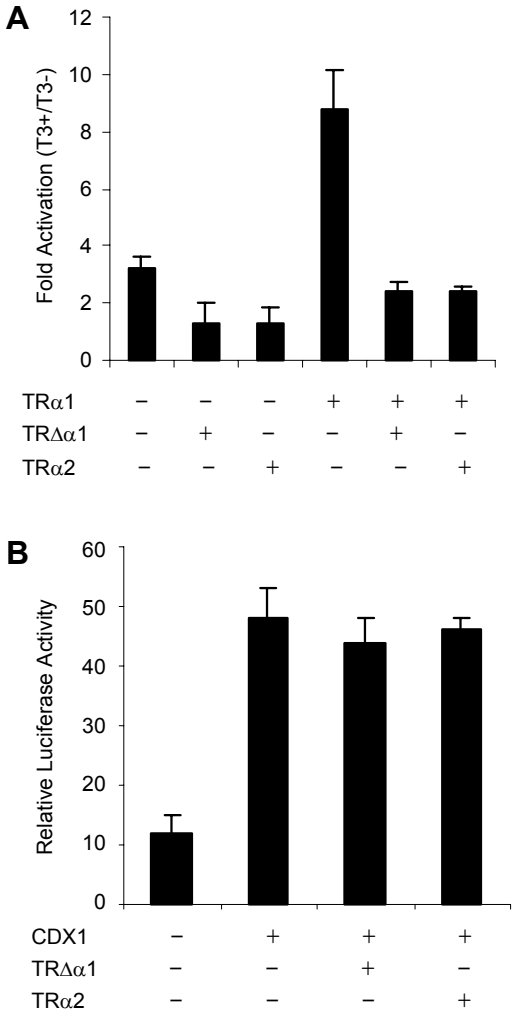
Fig. 1 (Munene)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8
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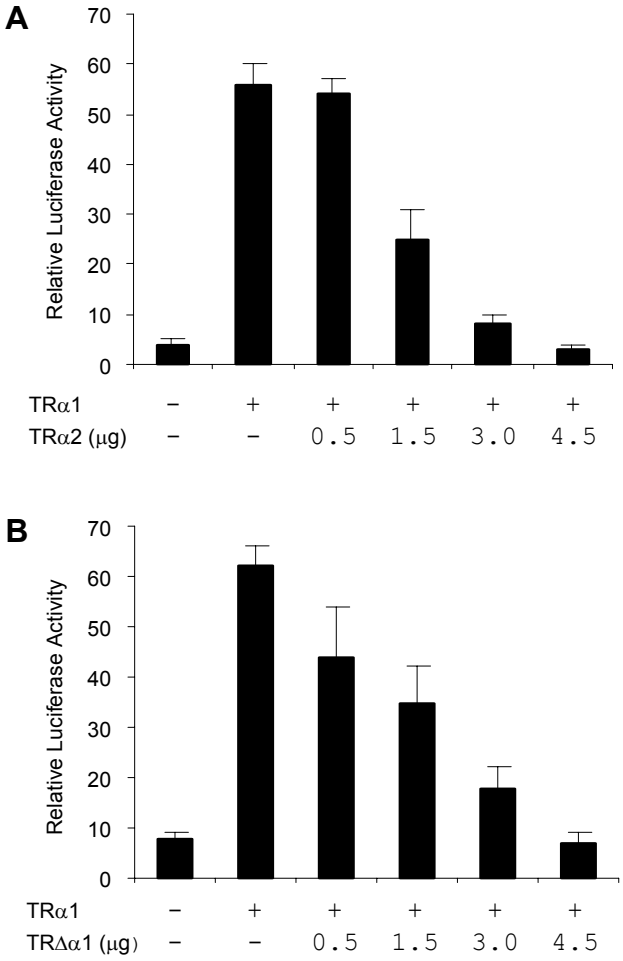
TRα2 − − − +

Fig. 2 (Munene)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TRα2 (μg) – – 0.5 1.5 3.0 4.5
B
Relative Luciferase Activity
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TRα1 – + + + + +
TRΔα1 (μg) – – 0.5 1.5 3.0 4.5

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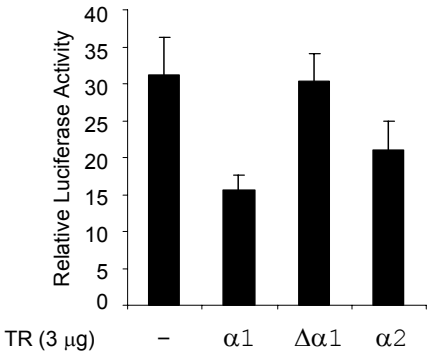
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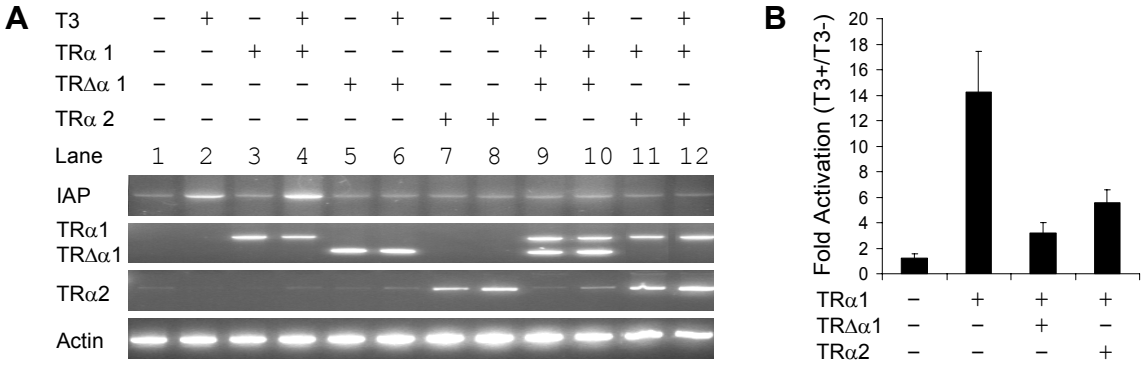
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Lane 1 2 3 4 5 6 7 8 9 10 11 12
IAP
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TRΔα1
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Fold Activation (T3+/T3-)
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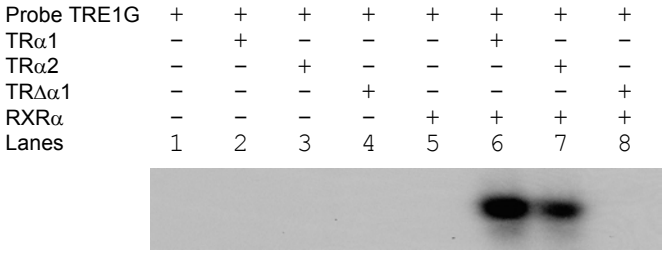

Figure legends

Fig. 1. Luciferase reporter assay data showing IAP regulation by the *bona-fide* and/or non-*bona-fide* TR isoforms. Caco-2 cells were transiently transfected with an IAP-luciferase reporter plasmid carrying 2.5 kb proximal IAP promoter region (see Materials and Methods). When indicated, the cells were also co-transfected with the plasmid expressing TRα1, TR$\Delta\alpha$1 and/or TRα2. Nonspecific plasmid DNA was used to maintain equal amounts of DNA in all transfection samples. Cells were treated +/- 100 nm T_3. Results are expressed in fold-activation (T_3^+/T_3^-) of relative firefly luciferase activity after normalization with *Renilla* luciferase activity from four independent experiments, and the values are expressed as mean ± SD ($P < 0.05$). **A:** The effects of non-*bona-fide* TR$\Delta\alpha$1 and TRα2 isoforms on *bona-fide* isoform TRα1-mediated IAP-Luciferase reporter gene expression. **B:** The effects of TR$\Delta\alpha$1 and TRα2 on Cdx1-mediated IAP gene expression.

Fig. 2. Luciferase reporter assay data showing dose-dependent effects of TR variants on TRα1-mediated IAP activation. Caco-2 cells were transiently transfected with the IAP-luciferase reporter plasmid carrying 2.5 kb proximal IAP promoter region (see Materials and Methods). Cells were also co-transfected with a fixed amount of TRα1, and varying amounts of the plasmid expressing TR$\Delta\alpha$1 or TRα2. Nonspecific plasmid DNA was used to maintain equal amounts of DNA in all transfection samples. Cells were treated +/- 100 nm T_3. Results are expressed in fold-activation (T_3^+/T_3^-) of relative firefly luciferase activity after normalization with *Renilla* luciferase activity from four independent experiments, and the values are expressed as mean ± SD ($P < 0.05$). **A:** The dose-dependent effects of TR$\Delta\alpha$1 on TRα1-mediated IAP gene expression. **B:** The dose-dependent effects of TRα2 on TRα1-mediated activation of the IAP gene.

Fig. 3. Luciferase reporter assay data showing effects of different TR isoforms on ligand-independent repression. Caco-2 cells were transiently transfected with an IAP-luciferase reporter plasmid carrying 2.5 kb proximal IAP promoter region (see Materials and Methods). When indicated, the cells were also co-transfected with the plasmid expressing TRα1, TR$\Delta\alpha$1 or TRα2. Nonspecific plasmid DNA was used to maintain equal amounts of DNA in all transfection samples. Cells were grown in the absence T_3. Results are expressed as relative firefly luciferase activity after normalization with *Renilla* luciferase activity from four independent experiments, and the values are expressed as mean ± SD ($P < 0.05$).

Fig. 4. RT-PCR data showing the regulation of the endogenous IAP gene. Caco-2 cells were transfected with the TRα1, TR$\Delta\alpha$1, and/or TRα2 expression plasmid, treated +/- 100 nm T_3. Total RNA was isolated 48 h after transfection from the untransfected as well as from transfected cells, and then cDNA was synthesized (see Materials and Methods). **A:** Semiquantitative RT-PCR analyses of IAP expression. Sequences of the related primers and PCR conditions are described in Materials and Methods. The PCR products were electrophoresed in agarose gels containing ethidium bromide. The gels were illuminated with UV light and then photographed. **B:** Real-time PCR analyses of IAP expression. Using the same cDNA (described above). Real-time RT-PCR was performed as described in Materials and Methods. Real-time PCR was performed in three independent experiments.

Fig. 5. Binding of TRα1, TR$\Delta\alpha$1, and TRα2 to the IAP-TRE analyzed by EMSA. Double-stranded oligonucleotide carrying the IAP-TRE was 5' end-labeled with ^{33}P, and used as the probe in EMSA. TRα1, TR$\Delta\alpha$1, and TRα2, and RXRα proteins were synthesized *in vitro*, and approximately 1 ng of each protein was used in each reaction (see Materials and Methods). The

protein-DNA complexes were separated by electrophoresing through a 5% nondenaturing polyacrylamide gel at 4º C. EMSA for each experiment was repeated more than three times, and similar results were obtained.